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(54) Title: CELLULAR TCR LIGAND ASSAY METHOD

(57) Abstract: The present invention provides an assay method comprising providing a plurality of test cells, which present a given TCR ligand, contacting the test cells with an excess of αβ T cell receptors (TCRs) which specifically recognise and bind to said TCR ligand, said TCRs having a Kd for their interaction with the said TCR ligand of 50 nM or less, said TCRs being labelled, or adapted to be labelled, with a detectable signal; separating non-cell-bound TCRs from the cells, in the case where the TCRs were adapted to be labelled rather than labelled, labelling the cell-bound TCRs with the detectable signal, then detecting and quantifying the label signals from one or a plurality of the cells, and estimating from the resultant signal quantity(ies) the average number of said TCR ligand presented per cell. The information gathered from such analyses may provide diagnostically and therapeutically useful information.

Cellular TCR ligand Assay Method

The invention relates to an assay method for estimating the average number per cell of molecules of a given TCR ligand on a sample of test cells. The assay relies on the use of αβ T cell receptors (TCRs) which specifically recognise and bind to said TCR ligand, said TCRs having a Kd for their interaction with the said TCR ligand of 50 nM or less.

Background to the Invention

Peptide-Major Histocompatibility Complexes (pMHCs)

pMHC molecules are one class of cell-bound TCR ligand. Almost all nucleated cells of higher vertebrates present MHC molecules. Such MHC expressing cells are known as antigen presenting cells (APCs). The MHC molecules of these APCs form a complex with peptide antigens so that the peptides are presented on the surface of the APCs as pMHCs. pMHCs are recognised by T cells via T cell receptors (TCRs) and a co-receptor expressed on the surface of the T cell. Binding of the MHC-peptide complex with the TCR and coreceptor transduces signals in the T cell that activate the cell, leading to a cellular immune response.

MHC molecules are divided into MHC Class I and MHC Class II molecules. The former require the CD8 co-receptor for T cell activation, and the latter require the CD4 coreceptor for T cell activation.

Native TCRs

As is described in, for example, WO 99/60120 TCRs mediate the recognition of specific Major Histocompatibility Complex (MHC)-peptide complexes by T cells and, as such, are essential to the functioning of the cellular arm of the immune system.

Antibodies and TCRs are the only two types of molecules which recognise antigens in a specific manner, and thus the TCR is the only receptor for particular peptide antigens presented in MHC, the alien peptide often being the only sign of an abnormality within a cell. T cell recognition occurs when a T-cell and an antigen presenting cell (APC) are in direct physical contact, and is initiated by ligation of antigen-specific TCRs with pMHC complexes.

The native TCR is a heterodimeric cell surface protein of the immunoglobulin superfamily which is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. TCRs exist in αβ and γδ forms, which are structurally similar but have quite distinct anatomical locations and probably functions. The MHC class I and class II ligands are also immunoglobulin superfamily proteins but are specialised for antigen presentation, with a highly polymorphic peptide binding site which enables them to present a diverse array of short peptide fragments at the APC cell surface.

The extracellular portion of native heterodimeric αβ and γδ TCRs consist of two polypeptides each of which has a membrane-proximal constant domain, and a membrane-distal variable domain. Each of the constant and variable domains includes an intra-chain disulfide bond. The variable domains contain the highly polymorphic loops analogous to the complementarity determining regions (CDRs) of antibodies. CDR3 of αβ TCRs interact with the peptide presented by MHC, and CDRs 1 and 2 of αβ TCRs interact with the peptide and the MHC. The diversity of TCR sequences is generated via somatic rearrangement of linked variable (V), diversity (D), joining (J), and constant genes

Functional α and γ chain polypeptides are formed by rearranged V-J-C regions, whereas β and δ chains consist of V-D-J-C regions. The extracellular constant domain has a membrane proximal region and an immunoglobulin region. There are single α and δ chain constant domains, known as TRAC and TRDC respectively. The β chain

constant domain is composed of one of two different β constant domains, known as TRBC1 and TRBC2 (IMGT nomenclature). There are four amino acid changes between these β constant domains, three of which are within the domains used to produce the single-chain TCRs displayed on phage particles of the present invention. These changes are all within exon 1 of TRBC1 and TRBC2: N_4K_5->K_4N_5 and F_{37}->Y (IMGT numbering, differences TRBC1->TRBC2), the final amino acid change between the two TCR β chain constant regions being in exon 3 of TRBC1 and TRBC2: V_1->E. The constant γ domain is composed of one of either TRGC1, TRGC2(2x) or TRGC2(3x). The two TRGC2 constant domains differ only in the number of copies of the amino acids encoded by exon 2 of this gene that are present.

The extent of each of the TCR extracellular domains is somewhat variable. However, a person skilled in the art can readily determine the position of the domain boundaries using a reference such as The T Cell Receptor Facts Book, Lefranc & Lefranc, Publ. Academic Press 2001.

Recombinant TCRs

The production of recombinant TCRs is beneficial as these provide soluble TCR analogues suitable for the following purposes:

- Studying the TCR / ligand interactions (e.g. pMHC for αβ TCRs)
- Screening for inhibitors of TCR-associated interactions
- Providing the basis for potential therapeutics

A number of constructs have been devised to date for the production of recombinant TCRs. These constructs fall into two broad classes, single-chain TCRs and dimeric TCRs, the literature relevant to these constructs is summarised below.

Single-chain TCRs (scTCRs) are artificial constructs consisting of a single amino acid strand, which like native heterodimeric TCRs bind to MHC-peptide complexes. Unfortunately, attempts to produce functional alpha/beta analogue scTCRs by simply linking the alpha and beta chains such that both are expressed in a single open reading

frame have been unsuccessful, presumably because of the natural instability of the alpha-beta soluble domain pairing.

Accordingly, special techniques using various truncations of either or both of the alpha and beta chains have been necessary for the production of scTCRs. These formats appear to be applicable only to a very limited range of scTCR sequences. Soo Hoo *et al* (1992) PNAS. **89** (10): 4759-63 report the expression of a mouse TCR in single chain format from the 2C T cell clone using a truncated beta and alpha chain linked with a 25 amino acid linker and bacterial periplasmic expression (see also Schodin *et al* (1996) Mol. Immunol. **33 (9)**: 819-29). This design also forms the basis of the m6 single-chain TCR reported by Holler *et al* (2000) PNAS. **97** (10): 5387-92 which is derived from the 2C scTCR and binds to the same H2-Ld-restricted alloepitope. Shusta *et al* (2000) Nature Biotechnology **18**: 754-759 and US 6,423,538 report using a murine single-chain 2C TCR constructs in yeast display experiments, which produced mutated TCRs with, enhanced thermal stability and solubility. This report also demonstrated the ability of these displayed 2C TCRs to selectively bind cells expressing their cognate pMHC. Khandekar *et al* (1997) J. Biol. Chem. **272** (51): 32190-7 report a similar design for the murine D10 TCR, although this scTCR was fused to MBP and expressed in bacterial cytoplasm (see also Hare *et al* (1999) Nat. Struct. Biol. **6** (6): 574-81). Hilyard *et al* (1994) PNAS. **91** (19): 9057-61 report a human scTCR specific for influenza matrix protein-HLA-A2, using a Vα-linker-Vβ design and expressed in bacterial periplasm.

Chung *et al* (1994) PNAS. **91** (26) 12654-8 report the production of a human scTCR using a Vα-linker-Vβ-Cβ design and expression on the surface of a mammalian cell line. This report does not include any reference to peptide-HLA specific binding of the scTCR. Plaksin *et al* (1997) J. Immunol. **158** (5): 2218-27 report a similar Vα-linker-Vβ-Cβ design for producing a murine scTCR specific for an HIV gp120-H-2D^{d} epitope. This scTCR is expressed as bacterial inclusion bodies and refolded *in vitro*.

A number of papers describe the production of TCR heterodimers which include the native disulphide bridge which connects the respective subunits (Garboczi, *et al.,* (1996), Nature **384**(6605): 134-41; Garboczi, *et al.,* (1996), J Immunol **157**(12): 5403-10; Chang *et al.,* (1994), PNAS USA **91**: 11408-11412; Davodeau *et al.*, (1993), *J. Biol. Chem.* **268**(21): 15455-15460; Golden *et al.*, (1997), J. Imm. Meth. **206**: 163-169; US Patent No. 6080840). However, although such TCRs can be recognised by TCR-specific antibodies, none were shown to recognise its native ligand at anything other than relatively high concentrations and/or were not stable.

In WO 99/60120, a soluble TCR is described which is correctly folded so that it is capable of recognising its native ligand, is stable over a period of time, and can be produced in reasonable quantities. This TCR comprises a TCR α or γ chain extracellular domain dimerised to a TCR β or δ chain extracellular domain respectively, by means of a pair of C-terminal dimerisation peptides, such as leucine zippers. This strategy of producing TCRs is generally applicable to all TCRs.

Reiter *et al*, Immunity, 1995, **2**:281-287, details the construction of a soluble molecule comprising disulphide-stabilised TCR α and β variable domains, one of which is linked to a truncated form of Pseudomonas exotoxin (PE38). One of the stated reasons for producing this molecule was to overcome the inherent instability of single-chain TCRs. The position of the novel disulphide bond in the TCR variable domains was identified via homology with the variable domains of antibodies, into which these have previously been introduced (for example see Brinkmann, *et al.* (1993), Proc. Natl. Acad. Sci. USA **90:** 7538-7542, and Reiter, *et al.* (1994) Biochemistry **33:** 5451-5459). However, as there is no such homology between antibody and TCR constant domains, such a technique could not be employed to identify appropriate sites for new inter-chain disulphide bonds between TCR constant domains.

Class I MHC

Class I MHC molecules are presented by almost all nucleated cells of higher vertebrates. These molecules generally present peptides that are derived from intracellular polypeptides and proteins.

Class I MHC is a dimeric protein complex consisting of a variable heavy chain and a constant light chain, β_2-microglobulin (β_2m). The heavy and light chains of Class I MHC molecules are made by ribosomes on the rough endoplasmic reticulum and then translocated to the lumen of the endoplasmic reticulum (ER). The peptides that are loaded by Class I MHC molecules are generated in the cytosol by proteosomes, before being transported into the lumen of the ER. Within the ER the MHC heavy and light subunits and a peptide combine to form a stable pMHC molecule which is then transported to the cell surface via the Golgi. The pMHC molecules are anchored in the cell membrane by the MHC heavy chain. Class I MHC presented peptides are usually 8-11 amino acids in length, depending on the degree of arching introduced in the peptide when bound in the MHC molecule. The binding cleft, which is formed by the membrane distal α1 and α2 domains of the MHC heavy chain, has "closed" ends, imposing quite tight restrictions on the length of peptide which can be bound.

β_2m is a polypeptide found free in serum, which is non-covalently associated with MHC Class I molecules at the cell surface and which can exchange in the MHC complex with other free β_2m molecules (Bernabeu, *et al. Nature* **308**: 642-5 (1984); Cook, *et al. J Immunol* **157**: 2256-61 (1996); Horig, *et al. Proc Natl Acad Sci U S A* **94**: 13826-31 (1997); Hyafil & Strominger, *Proc Natl Acad Sci U S A* **76**: 5834-8 (1979); Luscher, *et al. J Immunol* **153**: 5068-81 (1994); Parker, *et al. J Immunol* **149**: 1896-904 (1992); Smith, *et al. Proc Natl Acad Sci U S A* **89**: 7767-71 (1992)).

Class I pMHC molecules are recognised by $CD8^+$ cyto-toxic T cells (CTLs). This recognition leads to a cyto-toxic response by the T cell which leads to the killing of the APC and clonal expansion of the CTL.

The CD8 coreceptor of CTLs is expressed as either a αβ homodimer, or occasionally as an αα heterodimer protein consisting of extracellular immunoglobulin, membrane-proximal stalk, transmembrane and cytoplasmic domains. The native dimers have a molecular weight of 47 & 45 kDa respectively (*The Leucocyte Antigen Factsbook*, 2nd Ed., Barclay *et al*, (1997) Academic Press).

Class II MHC

Class II MHC molecules are only presented by specialised APCs including the interdigitating dendritic cells found in the T cell areas of the lymph nodes and spleen in large numbers; Langerhans cells in the skin; follicular dendritic cells in B cell areas of the lymphoid tissue; monocytes, macrophages and other cells of the monocyte/macrophage lineage; B cells and T cells; and a variety of other cells such as endothelial cells and fibroblasts which are not classical (specialised?) APCs but can act as such. Class II MHC molecules generally present peptides derived from polypeptides and proteins in the extracellular spaces. (*The HLA Factsbook*, Marsh *et al*, (2000), Academic Press)

Class II MHC is a membrane-bound 61-65kDa αβ heterodimeric protein complex consisting of two similar non-covalently associated chains (*The Leucocyte Antigen Factsbook*, 2nd Ed., Barclay *et al*, (1997) Academic Press). Class II HLA heavy and light chains are made by ribosomes on the rough endoplasmic reticulum and then translocated to the lumen of the endoplasmic reticulum (ER). The Class II MHC molecules are then assembled with a polypeptide, known as the invariant chain. The Class II MHC-invariant chain complexes are then transported via the Golgi to endocytic vesicles called the MHC Class II compartment. (MIIC) Within this compartment the invariant chain is degradable and replaced by a peptide derived from endocytosed material. (*The HLA Factsbook*, Marsh *et al*, (2000), Academic Press)

The peptides presented by Class II MHC molecules are generally 12-24 amino acids in length. The binding cleft is formed by the membrane distal $\alpha 1$ and $\beta 1$ domains of the MHC chains (*The HLA Factsbook*, Marsh *et al*, (2000) Academic Press).

Class II pMHC molecules are recognised by $CD4^+$ helper T cells. This recognition leads to either the production of cytokines which directly act on macrophages to enhance their killing rate or the stimulation of B cells to produce antibodies specific for the Class II MHC-loaded peptide.

The CD4 coreceptor of T helper cells is expressed as monomeric protein consisting of extracellular region made up of four immunoglobulin superfamily domains, transmembrane and cytoplasmic domains. The native protein has a molecular weight of 48.4 kDa (*The Leucocyte Antigen Factsbook*, 2nd Ed., Barclay *et al*, (1997) Academic Press).

CD1

CD1 antigens are also capable of functioning as cell-bound TCR ligands. CD1 antigens are MHC class I-related molecules whose genes are located on a different chromosome from the classical MHC class I and class II antigens. CD1 molecules are capable of presenting peptide and non-peptide (e.g. lipid, glycolipid) moieties to T cells in a manner analogous to conventional class I and class II-MHC-pep complexes. See, for example (Barclay et al, (1997) The Leucocyte Antigen Factsbook 2nd Edition, Academic Press) and (Bauer (1997) Eur J Immunol **27** (6) 1366-1373))

Altered peptide-MHC presentation by cancerous cells

APCs present pMHCs whether they are "healthy" or not. The MHC molecules of healthy cells will present "self" peptides and such complexes are "scanned" by T cells but do not normally elicit a T cell response.

As is known to those skilled in the art there are many mechanisms by which a "healthy" cell can become cancerous. These result in the cell overcoming the normal constraints on cell division. The transformation of a healthy cell to a cancerous include mutations within genes involved in the regulation of cell cycle and/or translocation events that lead to the up or down-regulation of gene expression.

These genetic changes may alter the repertoire of Class I MHC molecules on the surface of the cancer cells, such that altered peptides and/or unusual quantities of a self peptide are presented. This change in Class I pMHC can lead to recognition and killing of the cancerous cells by CTLs. However, some cancer cells that proliferate *in-vivo* do so by evading immune surveillance by means such as reducing the level of cell surface Class I pMHC expression. (Bubenik (2003) *Oncol Rep.* **10** (6) 2005-8) provides a recent review of Class I MHC down-regulation and its impact on immunotherapy. The number of cancer-specific pMHC molecules that are expressed on the surface of tumour cells is likely to vary according to cell –type and the individual. The present invention provides a means of estimating this variation either for a population of cancer cell or on a cell-by-cell basis. The information gathered from such analyses may provide diagnostically and therapeutically useful information.

Altered peptide-MHC presentation during infection

As is known to those skilled in the art, once a cell becomes infected by an intracellular pathogen the range of peptides presented on the Class I MHC molecules may be altered. Such infected cells normally present "foreign" peptides derived from the invading pathogen on their Class I MHCs as well as "self" peptides. The Class I foreign peptide-MHC are recognised by CTLs. There are many types of pathogen which are capable of invading human cells including, but not limited to, the following:

- Viruses, such as influenza, hepatitis B virus (HBV), Epstein –Barr virus (EBV) and human immunodeficiency Virus (HIV)
- Bacteria, such as mycoplasmas

- Protozoa, such as plasmodium and trypanosomes.

There are also many types of extracellular pathogen including, but not limited to bacteria, such as Staphylococcus aureus and Pseudomonas aeruginosa which are capable of causing disease in humans without invading cells. The presence of such extracellular pathogens generally provokes a humoral (antibody) based response. This response is facilitated by the presentation of peptides derived from the pathogen on the Class II MHC molecules of specialised APCs.

High Affinity TCRs

T cells mature in the thymus where they undergo at least two selection mechanisms, generally referred to as positive and negative selection. The structures of most, or all, TCRs are believed to share certain general architectural features (Chothia, *et al, Embo J* (1988) **7**: 3745-55) that provide a framework suitable for MHC/peptide binding by the variable complementarity determining regions (CDRs). Thus, most TCRs may have intrinsic affinity for MHC/peptide complexes (Chothia, *et al, Embo J* (1988) **7**: 3745-55). In the thymus, only TCRs with a certain minimal level of affinity for one of the MHC molecules to which they are presented (the "self" MHC molecules) will be positively selected. T cells with high affinity for one of the self MHC molecules will be negatively selected (Amsen & Kruisbeek. (1998). *Immunol Rev* **165**: 209-29. Sebzda, *et al* (1999). *Annu Rev Immunol* **17:** 829-74).

TCRs in the cellular immunity can be considered to be analogous to antibodies in the humoral immunity. Antibodies have been successfully used, either as therapeutic agents in their own right (e.g. Herceptin) or as targeting agents (e.g. mylotarg), and interest in this area continues to grow. Similar strategies could be devised using T cell receptors. Thus, soluble TCRs are useful, not only for the purpose of investigating specific TCR-pMHC interactions, but also as a diagnostic tool to detect infection, or to detect autoimmune disease markers, or to detect the efficacy of T cell vaccines. Soluble TCRs also have applications in staining, for example to stain cells for the

presence of a particular cancer or viral antigen presented in the context of the MHC. Similarly, soluble TCRs can be used to deliver a therapeutic agent, for example a cytotoxic compound or an immunostimulating compound, to cells presenting a particular antigen.

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However, two factors have hindered the exploitation of TCRs. Firstly, a generally applicable method for the production of soluble (i.e. non-membrane bound) T cell receptors have only been made available in the last few years. Secondly, the affinity of the T cell receptor for its specific pMHC ligand is much lower (K_D in the μM range) 10 than for antibodies (K_D in the nM range). This lower affinity of the TCR is thought to be a result of negative selection during development, and it is therefore probably not possible to find TCRs with high affinity for self-MHC-peptide complexes (Salzmann & Bachmann, *Molecular Immunology*, 1998, **35**:65-71).

15 Methods for *in vitro* selection of high affinity antibodies have been developed using phage display technology. In one particular method phage particles are caused to display a diverse library of mutated αβ TCRs which contain a non-native disulfide interchain bond. The phage-displayed d TCR library is the subjected to one or more round(s) of selection against a given pMHC in order to identify high affinity TCRs 20 clones specific for the given pMHC. (WO 2004/044004)

Yeast has also been investigated as a system for displaying scTCR and also for selecting stable and high affinity scTCR mutants. (US6,759,243, (Holler, *et al.* (2000) *Proc. Natl. Acad. Sci.* **97**, 5387-5392; Shusta, *et al.* (2000) *Nature Biotechnology* **18**, 25 754-759; Shusta, *et al.* (1999) *J. Mol. Biol.* **292**, 949-956; Kieke, *et al.* (1999) *Proc. Natl. Acad. Sci.* **96**, 5651-5656)). These similar studies report an increase in affinity from a K_D of 1.5 μM to 9 nM, an increase of 160-fold for the murine 2C TCR. However, there is currently insufficient data available to prove whether this approach is generally applicable.

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Other techniques for the production of high affinity TCRs have been directed to altering the amino acid sequence of a TCR so that it can bind more strongly to its ligand. This approach was originally applied to the production of high affinity monoclonal antibodies, and attempts to mutate antibodies, based on modelling of the antibody/hapten interface, produced two to three fold increases in their affinity by altering the amino acid sequence in the CDR3 regions. (Reichmann, *et al*, (1992) *J. Mol. Biol.* **224,** (4) 913-918).

A further study (Manning *et al.,* (1998) *Immunity* **8** (4): 413-25) utilised single-point alanine scanning to investigate binding of the murine 2C TCR to the QL9 / L^d complex. This study noted that 2 amino acids in each of the CDR2 loops elicited equivalent or slightly improved binding when replaced by alanine. (53α Asp, 54α Pro, 55β Thr and 56β Glu) A later study (Manning *et al.*, (1999) *J Exp Med* **189** (3): 461-70) by the same group carried out further single-point and combinations of alanine scanning mutations to investigate the binding contribution of several amino acids in the variable domain, including the CDR2 loops, of the 2C murine TCR. Mutant 2C TCR containing single alanine substitutions in both the TCR β CDR2 and TCR α CDR3 loops demonstrated an approximately 2-fold increase in affinity for the QL9 / L^d complex and slower association and disassociation properties. (K_D = 3.2 μM (WT) 2.54μM (mutant), k_{off} = 19 x 10^{-3} S^{-1} (WT) and 5.9 x 10^{-3} S^{-1} (mutant) This study also noted that despite the only 2-fold increase in affinity achieved by directed site-specific mutagenesis, these results indicate the promise in using more sophisticated techniques to obtain even greater improvements in TCR affinity.

It is known that TCRs can exhibit higher affinities for allogenic pMHCs than syngeneic pMHCs. For example, the affinity of the 2C TCR for its natural ligand, dEV8 peptide (EQYKFYSV), presented by MHC H2-K^b has been determined as K_D = 84.1 x 10^6 M^{-1} ± 12.0 μM (Garcia, *et al*, (1997) *Proc. Natl. Acad. Sci. USA.* **94:** 13838-13843). This compares to a K_D of 1.5 x 10^7 M^{-1} observed for the 2C T cell clone binding to the allogeneic ligand, QL9 peptide (QLSPFPFDL) presented by MHC H2-L^d. (Sykulev, *et al*, (1994) *Proc. Natl. Acad. Sci. USA.* **91,** p11487-11491).

WO 2004/044004 discloses generally applicable proteinaceous particle (preferable phage particle) display-based methods for the production of high affinity αβTCRs.

Quantitation of the expression of specific pMHC molecules on the surface of cells

There have been a number of studies reporting methods suitable for the quantitation of the expression of specific pMHC molecules on the surface of cells.

One study (Lev *et al.*, (2002) *Cancer Res.*, **62** 3184-3194) details the use of pMHC-specific antibody Fab monomers for labelling cells transfected to express the Telomerase catalytic subunit (hTERT) and tumour cells which naturally expressed this protein. The level of pMHC expression was determined by FACS using an anti-human Fab labelled with fluorescein isothiocyanate (FITC) to provide the fluorescent signal. It should be noted that the number of FITC molecules which bind to a given Fab fragments may vary across a population of such labelled molecules, making the enumeration of a specific pMHC on a single cell using a FITC labelling method unreliable. Furthermore, FITC is not a strong enough fluorophore to allow microscopy-based detection of a single FITC molecule.

A similar study (Cohen *et al.*, (2003) *J. Immunology* **170** 4349-4361) details the use of HLA-A2-Tax specific phycoerthyrin (PE) labelled antibody Fab fragment monomers and tetramers to analyse the expression of HLA-A2-Tax label peptide-pulsed and unpeptide-pulsed cells respectively by FACS. This study also details the use of HLA-A2-Tax specific Horseradish peroxidase (HRP) antibody Fab fragment tetramers for immunohistochemical studies of HLA-A-Tax expression. This study states that the enumeration of as little as 100 specific pMHC molecules / cell was achieved. The authors notes that this is approximately the level of pMHC expression required to elicit nuclear responses such as cytokine expression by CTLs but approximately 10-fold higher than the level of pMHC expression required for CTL-mediated cell lysis. This study also notes the possibly use of confocal microscopy for studying the

intracellular sites of pMHC interaction and trafficking and that "In-situ localisation of peptide-MHC TCR ligands would be especially valuable in characterising the cell-cell interactions involved in initiation, propagation, and maintenance of T cell immune responses."

Three similar studies (Irvine *et al.*, (2002) *Nature* **419** 845-849), (Purhboo *et al.*, (2004) *Nature Immunology* **5** (5) 524-530) and (Li *et al.*, (2004) *Nature Immunology* **8** (5) 791-799) disclose a 3-dimensional fluorescence microscopy-based method of visualising specific pMHC molecules on APCs during the interaction of these APCs with T cells. The method relies on pulsing APCs with a reagent comprising Class I or Class I MHC-loaded peptides elongated by a C or N-terminal peptide linker terminated with biotin. The peptide pulsed cells are then contacted with PE-labelled streptavidin in order to form the complete MHC-loaded, fluorescent-labelled, elongated peptide.

US patent application no. US 2001/0006782 discloses methods for the diagnosis of Endometriosis based on comparing the HLA A/B/C complex expression levels of healthy and potentially diseased cells. The disclosed methods depend on contacting the healthy and potentially diseased cells with HLA A/B/C specify antibodies, of fragments thereof.

Finally, the applicant's published application (WO 03/020763) contains the following statements:

"Soluble TCRs are useful, not only for the purpose of investigating specific TCR-pMHC interactions, but also potentially as a diagnostic tool to detect infection, or to detect autoimmune disease markers. Soluble TCRs also have applications in staining, for example to stain cells for the presence of a particular peptide antigen presented in the context of the MHC".

"In tetrameric TCR formed using biotinylated heterodimers, fluorescent streptavidin (commercially available) can be used to provide a detectable label. A fluorescently-labelled tetramer is suitable for use in FACS analysis, for example to detect antigen presenting cells carrying the peptide for which the TCR is specific."

Brief Description of the Invention

The present invention provides an assay method comprising providing a plurality of test cells, which present a specific TCR ligand, contacting the test cells with an excess of αβ T cell receptors (TCRs) which specifically recognise and bind to said TCR ligand, said TCRs having a Kd for their interaction with the said TCR ligand of 50 nM or less, said TCRs being labelled, or adapted to be labelled, with a detectable signal; separating non-cell-bound TCRs from the cells, in the case where the TCRs were adapted to be labelled rather than labelled, labelling the cell-bound TCRs with the detectable signal, then detecting and quantifying the label signals from one or a plurality of the cells, and estimating from the resultant signal quantity(ies) the average number of said TCR ligands presented per cell.

Detailed Description of the Invention

In one broad aspect the invention provides an assay method comprising providing a plurality of test cells, which present a specific TCR ligand, contacting the test cells with an excess of αβ T cell receptors (TCRs) which specifically recognise and bind to said TCR ligand, said TCRs having a Kd for their interaction with the said TCR ligand of 50 nM or less, said TCRs being labelled, or adapted to be labelled, with a detectable signal; separating non-cell-bound TCRs from the cells, in the case where the TCRs were adapted to be labelled rather than labelled, labelling the cell-bound TCRs with the detectable signal, then detecting and quantifying the label signals from one or a plurality of the cells, and estimating from the resultant signal quantity(ies) the average number of said TCR ligands presented per cell.

Further preferred embodiments of the invention are provided wherein the αβ TCR has a Kd for their interaction with the said TCR ligand of 20 nM or less, more preferably 10nM or less, or most preferably 5 nM or less. Surface Plasmon Resonance (SPR) – based methods are suitable for determining the Kd for the interaction between the αβ TCR and the said TCR ligand. Example 3 herein provides a detailed description of a Biacore®-based method for carrying out such determinations.

Further embodiments of the present invention are provided wherein the TCR ligand is a Class I pMHC, or a Class II pMHC or a CD1-antigen.

In another embodiment of the invention the label signals are detected and quantified cell by cell.

The detectable signal may be produced by the spontaneous or excited emission of any form of electromagnetic wave including, but not limited to, gamma rays, visible or ultraviolet light. Alternatively, the detectable signal may be produced by the emission of a particle including, but not limited to α or β particles.

In a preferred embodiment of the invention the detectable signal is fluorescence. There are a number of fluorescent molecules that can be of use in the present invention, including, but not limited to, phycobiliproteins. Phycoerythrin (PE) is a particularly preferred phycobiliprotein for use in the present invention. In general, it is preferred that the fluorescent molecules utilised comprise a known number of fluorophores and have extinction coefficients and/or quantum yields in the order of $1.96 \times 10^6 - 2.4 \times 10^6$ M^{-1} cm^{-1} and 0.84 – 0.98 respectively.

In the case where the detectable signal is fluorescence, the label signals may be detected by 3-dimensional fluorescence microscopy and quantified by counting individual fluorescent signals. As is known to those skilled in the art, 3-dimensional fluorescence microscopy involves subjecting the specimen under investigation to light of the appropriate wavelength to excite the fluorophores utilised. This excitation of the

fluorophores causes them to emit light of a different, longer, wavelength. The microscope will reveal any fluorescence that occurs on, or very near to, the focal plane as a bright focussed spot, which is then recorded photographically. This process of excitation and emission is repeated through a range of focal planes until the entire specimen has been imaged. The images produced by this process can then be assessed in order to provide a count of the total number of fluorescent signals present on the specimen. As stated above, (Irvine *et al.*, (2002) *Nature* **419** 845-849), (Purhboo *et al.*, (2004) *Nature Immunology* **5** (5) 524-530) and (Li *et al.*, *Nature Immunology* **8** (5) 791-799) provide details relating to the 3-dimensional fluorescence microscopy of APCs.

For contacting with the TCR, the test cells may be in suspension or adhered to a substrate.

In some, perhaps most, cases, a few of the TCR ligand-specific TCRs which are contacted with the test cells may stick non-specifically to the cell surface. It may be desirable for certain applications to compensate for such non-specific "background" binding. In such cases, the required estimated average may adjusted by subtracting a number representing the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand. For example, the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand may be derived from one or more control assays wherein the method of the invention is repeated except that

(a) an analogous TCR which does not recognise the said TCR ligand is substituted for the cognate TCR, the resultant estimate being taken as representing the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand; and/or

(b) cells of the same cell classification but which do not present the said TCR ligand are substituted for the test cells, the resultant estimate being taken as representing the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand.

As referred to above, methods are known for the preparation of both single-chain TCRs (scTCRs) and dimeric TCRs (dTCRs), for utilisation in the current method. The manner of preparation of the TCR is not critical to the performance of the invention. However the soluble αβ dTCRs constructs containing a non-native disulfide interchain bond as disclosed in WO 03/020763 are particularly preferred and the preferred method for generating high affinity variants of TCRs specific for a given TCR ligand is selection from a diverse library of phage particles displaying such TCRs as disclosed in WO 2004/044004.

As stated above the high affinity TCR utilised in the present invention may either be labelled, or adapted to be labelled, with a detectable signal. There are many suitable methods, known to those skilled in the art of antibody labelling, by which such a detectable signal can be associated with said high affinity TCR. These include, but are not limited to, the following:

Biotin / streptavidin –mediated labelling – these methods rely on the high affinity of these two bacterial molecules for each other in order to facilitate the association of the high affinity TCR and detectable label. Briefly the high affinity TCR is biotinylated, using for instance the methods disclosed in WO 03/020763, and then contacted with a streptavidin-detectable label conjugate. These methods of association can be employed either before or after the high affinity TCR is contacted with the APCs. Example 2 herein provides details of a method for the formation of such a biotin-streptavidin mediated conjugate post contacting the high affinity TCR with the APCs. As is known to those skilled in the art there are a number of molecules that are a number streptavidin analogues such as extravidin, neutravidin and avadin which may be used to replace streptavidin.

Direct polypeptide fusion – these method rely on the production of a fusion protein comprising one chain of a high affinity dTCR or a high affinity scTCR and the polypeptide utilised as the detectable signal. In the case of a fusion protein comprising one chain of a dTCR fused to the polypeptide utilised as the detectable label a

subsequent refolding is required in order to form the dTCR-detectable label. This step involves the in-vitro refolding of the TCR chain-detectable label fusion protein with the remaining TCR chain. These methods of association can only be employed prior to contacting the high affinity TCR with the APCs.

Disulfide bond-mediated detectable label association – These methods rely on using a disulfide bond to facilitate the association between the high affinity TCR and the detectable label. WO 03/020763 details a method for the production of a soluble dTCR incorporating an unpaired cysteine residue at the C-terminus of either of the TCR chains. The polypeptide utilised as the detectable signal is then similarly altered to incorporate an unpaired cysteine reside at the C or N-termini. The modified high affinity TCR and polypeptide are then contacted in-vitro under conditions suitable for the formation of the disulfide bond-mediated association. These methods of association are only suited to the formation of an association between the high affinity TCR and the polypeptide utilised as the detectable signal prior to contacting the high affinity TCR with the APCs.

Multiple histidine tags – As is known to those skilled in the art tags containing multiple histidine residues can be added to the termini of polypeptides and proteins in order to facilitate the association of metal ions thereto. Hexa-histidine tags are the most commonly used example of such tags and these are generally used to facilitate the association of divalent metal cations such as Ni^{2+} or Co^{2+} with the polypeptides. In the context of the present invention multiple histidine tags may be utilised to facilitate the association of a radioactive metal cation to the high affinity TCR. These methods of association are only suited to the formation of an association between the high affinity TCR and the metal ion utilised as the detectable signal prior to contacting the high affinity TCR with the APCs.

Metal chelates – As is also known to those skilled in the art it is possible to attach chelating agents to polypeptides which can "trap" metal ions such as lanthanides. These lanthanide ions can have fluorescent and/or radioactive properties.

Methods of estimating TCR ligands on the surface of a cell

Example 2 herein details a preferred 3-dimensional fluorescence microscopy-based method of estimating the per cell average number of a given TCR ligand on the surface of a cell. However, the quantification of the signal from the labelled TCR ligand-bound TCRs provides a quantity related to the number of TCRs bound per cell, and therefore the required estimate is obtainable from that quantity. Quantification of course depend on the nature of the signal, but two alternatives to the microscopy-based methods are:

(i) Fluorescence-activated cell sorting (FACs), which allows the estimation of the average number of a given TCR ligand presented per cell for a population of cells.

(ii) Scintillation counting by which a detectable signal produced by radio-labelled TCRs may be detected, and which also allows the estimation of the average number of a given TCR ligand presented per cell for a population of cells.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

Examples

The invention is further described in the following examples, which do not limit the scope of the invention in any way.

Reference is made in the following to the accompanying drawings in which:

Figure 1a details the DNA sequence encoding the soluble Clone 134 A6 TCR α chain. This DNA sequence comprises an introduced non-native cysteine codon to facilitate disulfide bond-mediated interchain pairing of the expressed soluble TCR, and the native A6 TCR α variable domain. The inserted cysteine codon is shaded.

Figure 1b details the amino acid sequence of the soluble Clone 134 A6 TCR α chain. This amino sequence comprises an introduced non-native cysteine to facilitate disulfide bond-mediated interchain pairing of the soluble dTCR, and the native A6 TCR α variable domain. The inserted cysteine is shaded.

Figure 2a details the DNA sequence encoding the soluble Clone 134 A6 TCR β chain. This DNA sequence comprises an introduced non-native cysteine codon to facilitate disulfide bond-mediated interchain pairing of the expressed soluble dTCR and a mutated A6 TCR β variable domain. The inserted cysteine codon is shaded and the variable domain mutations are shown in bold.

Figure 2b details the amino acid sequence of the soluble Clone 134 A6 TCR β chain. This amino sequence comprises an introduced non-native cysteine to facilitate disulfide bond-mediated interchain pairing of the soluble dTCR and a mutated A6 TCR β variable domain. The inserted cysteine is shaded and the variable domain mutations are shown in bold.

Figure 3 illustrates the imaging of individual Streptavidin-PE/TCR complexes on APCs (J82 cancer cells transfected with a minigene for the Tax_{11-19} epitope) stained with the high affinity Clone 134 A6 TCR. The fluorescent images represents the *z*-stacks corresponding to the bottom plasma membrane (in contact with the chamber-slide).

Figure 4 illustrates the quantification of HLA/Tax_{11-19} complexes on peptide pulsed cells using high affinity TAXwtc134 TCR. T2 cells were pulsed with indicated concentrations of Tax_{11-19} peptide.

Figures 5a and 5b provide the amino acid sequences of the α and β chains of the high-affinity c58c61 1G4 TCR containing an introduced non-native disulfide interchain bond respectively The high affinity inducing mutated amino acids are underlined and the introduced cysteine codon are highlighted.

Figures 6a and 6b provide DNA sequences encoding the α and β chains of the high-affinity c58c61 1G4 TCR containing an introduced non-native disulfide interchain bond respectively. The high affinity inducing mutations are in bold and the introduced cysteine codon are highlighted.

Figure 7 illustrates the average number of SLLMWITQC-HLA-A*0201 complexes detected on the surface of individual SK-Mel-37, Mel 624 and Mel 526 tumour cells.

Example 1 - Production of high affinity HLA-A2- Tax specific TCR

Generic methods for the production of high affinity TCRs are disclosed in WO 2004/044004. In order to carry out the experiments described below the following biotinylated high affinity TCR was prepared:

The HLA-A2-Tax specific soluble clone 134 A6 TCR containing a non-native disulfide interchain bond was produced. The DNA and amino acid sequences of this soluble dTCR are provided in Figures 1 and 2 respectively. WO 2004/044004 discloses in detail the production of this particular high affinity soluble TCR. The TCR biotinylation methods disclosed in WO 03/020763 are appropriate for producing this soluble biotinylated TCR.

Example 2 - Quantification of cell surface TCR ligands by fluorescence microscopy using high affinity monoclonal TCR

The number of HLA-A2 complexed Tax_{11-19} antigens on target cells was determined (on the assumption that one fluorescence signal relates to a single labelled TCR bound to its cognate pMHC ligand on the surface of the target cell) by single molecule fluorescence microscopy using the high-affinity Clone 134 A6 TCR. This was facilitated by using biotinylated TCR to target antigen-pulsed or antigen-expressing antigen presenting cells and subsequent labelling of cell-bound TCR by streptavidin-R phycoerythrin (PE) conjugates. Individual PE molecules were then imaged by 3-dimensional fluorescence microscopy.

Staining of cells in suspension. T2 Cells were pelletted by centrifugation for 5 min at 14000 rpm (Megafuge 1.0, Hereaus). Where appropriate, pelleted cells were resuspended at 10^5-10^6 cells ml^{-1} in R10 medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, penicillin/streptomycin) and pulsed with antigen (10^{-5} – 10^{-10} M^{-1}) for at least 90 min at 37°C. Cells were washed twice by pelleting in 10 ml calcium and magnesium free phosphate buffered saline (PBS; Gibco), and the final pellet resuspended in 200 µl of TCR solution (10 µg ml^{-1} TCR in PBS containing 0.5% bovine serum albumin (BSA; Promega). Cells were left at 4°C for 30 min. Cells were washed twice by pelleting in 10 ml PBS. Cells were resuspended in 200 µl of streptavidin-PE (Pharmingen) solution (10 µg ml^{-1} streptavidin-PE in PBS containing 0.5% BSA). Cells were left in the dark at room temperature for 20 min. Cells were washed twice by pelleting in 10 ml PBS. Cells were resuspended at $5x10^5$ cells ml^{-1} in imaging media (R10 without phenol red). Cells were allowed to settle onto chamber well slides (Labtech), and imaged by fluorescence microscopy. (Excitation 565nm / Emission 575nm)

Staining of adherent cells. Cells were plated into chamber well slides and allowed to adhere overnight in incubator. (37°C, 5% CO_2) Media was removed and replaced with fresh R10. Where appropriate, cells were pulsed with peptide for at least 90 min at 37°C. Media was removed, and cells washed twice with 500 µl of PBS supplemented with 400 µM $MgCl_2$ (PBS/Mg). Cells were incubated in 200 µl of TCR solution (10 µg ml^{-1} TCR in PBS/Mg containing 0.5% BSA) for 30 min at 4°C. TCR solution

was removed, and cells were washed three times with 500 µl of PBS/Mg. Cells were incubated in 200 µl of streptavidin-PE solution (10 µg ml^{-1} streptavidin-PE in PBS/Mg containing 0.5% BSA) at room temperature in the dark for 20 min. Streptavidin-PE solution was removed and cells were washed five times with 500 µl of PBS/Mg. Wash media was removed, and cells kept in 400 µl of imaging media before imaging by fluorescence microscopy.

Fluorescence microscopy. Fluorescent microscopy was carried out using an Axiovert 200M (Zeiss) microscope with a 63x Oil objective (Zeiss). A Lambda LS light source containing a 300W Xenon Arc lamp (Sutter) was used for illumination, and light intensity was reduced to optimal levels by placing a 0.3 and a 0.6 neutral density filter into the light path. Excitation and emission spectra were separated using a TRITC/DiI filter set (Chroma). Cells were imaged in three dimensions by *z*-stack acquisition (21 planes, 1 µm apart). Image acquisition and analysis was performed using Metamorph software (Universal Imaging) as described (Irvine *et al.*, Nature (419), p845-9, and Purbhoo *et al.*, Nature Immunology (5), p524-30.).

Results

As demonstrated by figures 3 and 4 the above method was used successfully to image HLA-A2- Tax complexes on the surface of both peptide-pulsed T2 cells and J82 cancer cells transfected with a minigene for the Tax_{11-19} peptide.

Example 3 – BIAcore surface plasmon resonance characterisation of a high affinity A6 TCR binding to HLA-A2 Tax.

A surface plasmon resonance biosensor (BIAcore 3000™) was used to analyse the binding of the high affinity clone 134 A6 TCR to the HLA-A2 Tax ligand. This was facilitated by producing pMHC complexes (described below) which were immobilised to a streptavidin-coated binding surface in a semi-oriented fashion, allowing efficient testing of the binding of a soluble T-cell receptor to up to four different pMHC

(immobilised on separate flow cells) simultaneously. Manual injection of HLA complex allows the precise level of immobilised class I molecules to be manipulated easily.

Biotinylated class I HLA-A2 tax complexes were refolded *in vitro* from bacterially-expressed inclusion bodies containing the constituent subunit proteins and synthetic peptide, followed by purification and *in vitro* enzymatic biotinylation (O'Callaghan *et al.* (1999) *Anal. Biochem.* **266**: 9-15). HLA-heavy chain was expressed with a C-terminal biotinylation tag which replaces the transmembrane and cytoplasmic domains of the protein in an appropriate construct. Inclusion body expression levels of ~75 mg/litre bacterial culture were obtained. The HLA light-chain or β2-microglobulin was also expressed as inclusion bodies in *E.coli* from an appropriate construct, at a level of ~500 mg/litre bacterial culture.

E. coli cells were lysed and inclusion bodies were purified to approximately 80% purity. Protein from inclusion bodies was denatured in 6 M guanidine-HCl, 50 mM Tris pH 8.1, 100 mM NaCl, 10 mM DTT, 10 mM EDTA, and was refolded at a concentration of 30 mg/litre heavy chain, 30 mg/litre β2m into 0.4 M L-Arginine-HCl, 100 mM Tris pH 8.1, 3.7 mM cystamine, mM cysteamine, 4 mg/ml peptide (e.g. tax 11-19), by addition of a single pulse of denatured protein into refold buffer at < 5°C. Refolding was allowed to reach completion at 4°C for at least 1 hour.

Buffer was exchanged by dialysis in 10 volumes of 10 mM Tris pH 8.1. Two changes of buffer were necessary to reduce the ionic strength of the solution sufficiently. The protein solution was then filtered through a 1.5μm cellulose acetate filter and loaded onto a POROS 50HQ anion exchange column (8 ml bed volume). Protein was eluted with a linear 0-500 mM NaCl gradient. HLA-A2-peptide complex eluted at approximately 250 mM NaCl, and peak fractions were collected, a cocktail of protease inhibitors (Calbiochem) was added and the fractions were chilled on ice.

Biotinylation tagged HLA-A2 complexes were buffer exchanged into 10 mM Tris pH 8.1, 5 mM NaCl using a Pharmacia fast desalting column equilibrated in the same

buffer. Immediately upon elution, the protein-containing fractions were chilled on ice and protease inhibitor cocktail (Calbiochem) was added. Biotinylation reagents were then added: 1 mM biotin, 5 mM ATP (buffered to pH 8), 7.5 mM MgCl2, and 5 μg/ml BirA enzyme (purified according to O'Callaghan *et al.* (1999) *Anal. Biochem.* **266**: 9-15). The mixture was then allowed to incubate at room temperature overnight.

Biotinylated HLA-A2 complexes were purified using gel filtration chromatography. A Pharmacia Superdex 75 HR 10/30 column was pre-equilibrated with filtered PBS and 1 ml of the biotinylation reaction mixture was loaded and the column was developed with PBS at 0.5 ml/min. Biotinylated HLA-A2 complexes eluted as a single peak at approximately 15 ml. Fractions containing protein were pooled, chilled on ice, and protease inhibitor cocktail was added. Protein concentration was determined using a Coomassie-binding assay (PerBio) and aliquots of biotinylated HLA-A2 complexes were stored frozen at –20°C. Streptavidin was immobilised by standard amine coupling methods.

The interactions between the high affinity A6 Tax TCR containing a novel inter-chain bond and the HLA-A2 Tax complex or an irrelevant HLA-A2 NY-ESO combination, the production of which is described above, were analysed on a BIAcore 3000™ surface plasmon resonance (SPR) biosensor. SPR measures changes in refractive index expressed in response units (RU) near a sensor surface within a small flow cell, a principle that can be used to detect receptor ligand interactions and to analyse their affinity and kinetic parameters. The probe flow cells were prepared by immobilising the individual HLA-A2 peptide complexes in separate flow cells via binding between the biotin cross linked onto β2m and streptavidin which have been chemically cross linked to the activated surface of the flow cells. The assay was then performed by passing sTCR over the surfaces of the different flow cells at a constant flow rate, measuring the SPR response in doing so. Initially, the specificity of the interaction was verified by passing soluble A6 TCR at a constant flow rate of 5 μl min-1 over four different surfaces; one coated with ~1000 RU of HLA-A2 Tax complex, the second

coated with ~1000 RU of HLA-A2 NY-ESO complex, and two blank flow cells coated only with streptavidin.

The Kd for the interaction between the high affinity Clone 134 A6 TCR and its cognate HLA-A2-Tax pMHC was determined to be approximately 5 nM. The Kd for the same interaction using a wild-type soluble A6 TCR is 1-2 μM.

Example 4- Staining of SK-Mel-37, Mel 624 and Mel 526 tumour cells with a high affinity biotinylated c58c61 1G4 TCR containing an introduced non-native disulfide interchain bond.

The number of HLA-A*0201 complexes loaded with the NY-ESO-1 derived SLLMWITQC peptide on the surface of SK-Mel-37, Mel 624 and Mel 526 tumour cells was determined (on the assumption that one fluorescence signal relates to a single labelled TCR bound to its cognate pMHC ligand on the surface of the target cell) by single molecule fluorescence microscopy using the high-affinity biotinylated c58c61 1G4 TCR containing an introduced non-native disulfide interchain bond. (The amino acid sequence of the α and β chains of this soluble TCR are shown in Figures 5a and 5b respectively, and the corresponding DNA sequences are shown in Figures 6a and 6b respectively) This was facilitated by using the biotinylated TCR to target the adherent antigen-expressing SK-Mel-37, Mel 624 and Mel 526 tumour cells and subsequent labelling of cell-bound TCR by streptavidin-R phycoerythrin (PE) conjugates. Individual PE molecules were then imaged by 3-dimensional fluorescence microscopy.

Staining of tumour cells. The tumour cells were plated into chamber well slides and allowed to adhere overnight in incubator. (37°C, 5% CO_2) Media was removed and replaced with fresh R10. Media was removed, and cells washed twice with 500 μl of PBS supplemented with 400 μM $MgCl_2$ (PBS/Mg). Cells were incubated in 200 μl of TCR solution (10 μg ml^{-1} TCR in PBS/Mg containing 0.5% BSA) for 30 min at room temperature. The TCR solution was removed, and cells were washed three times with

500 µl of PBS/Mg. Cells were incubated in 200 µl of streptavidin-PE solution (10 µg ml^{-1} streptavidin-PE in PBS/Mg containing 0.5% BSA) at room temperature in the dark for 20 min. Streptavidin-PE solution was removed and cells were washed five times with 500 µl of PBS/Mg. Wash media was removed, and cells kept in 400 µl of imaging media before imaging by fluorescence microscopy.

The effect of interferon γ (IFNγ) on the number of SLLMWITQC-HLA-A*0201 complexes on the surface of these tumour cells was investigated by incubation of the cells in 1000 units/ml IFNγ for 72 hours prior to staining.

A number of control stainings were also carried out. Firstly, an irrelevant mTCR staining (the 134 A6 TCR used in Example 2) was carried out using the same concentration (10 µg ml^{-1} TCR in PBS/Mg containing 0.5% BSA) of this TCR as used for the cognate high affinity c58c61 1G4 TCR. Secondly, 500 µg/ml non-biotinylated soluble c58c61 1G4 TCR was added into the TCR staining solution in order to study the ability of this solule TCR to compete for binding to the cognate SLLMWITQC-HLA-A2 complexes. Finally, a "soluble TCR-free" control staining using only streptavidin-PE was carried out.

Fluorescence microscopy. Fluorescent microscopy was carried out using an Axiovert 200M (Zeiss) microscope with a 63x Oil objective (Zeiss). A Lambda LS light source containing a 300W Xenon Arc lamp (Sutter) was used for illumination, and light intensity was reduced to optimal levels by placing a 0.3 and a 0.6 neutral density filter into the light path. Excitation and emission spectra were separated using a TRITC/DiI filter set (Chroma). Cells were imaged in three dimensions by *z*-stack acquisition (21 planes, 1 µm apart). Image acquisition and analysis was performed using Metamorph software (Universal Imaging) as described (Irvine *et al.,* Nature (419), p845-9, and Purbhoo *et al.,* Nature Immunology (5), p524-30.).

Results

As demonstrated by Figure 7 the above method was used successfully to image high affinity 1G4 TCR bound to SLLMWITQC-HLA-A*0201 antigens on the surface of Mel 526, Mel 624 and SK-Mel-37 cancer cells. The results produced indicate that the number of SLLMWITQC-HLA-A*0201 complexes present on the surface of these cells is greatest on the Mel 624 cells and lowest of the Mel 526 cells.

The IFNγ pre-incubation slightly increased the number of the SLLMWITQC-HLA-A*0201 complexes present on the surface of the SK-Mel -37 cells. The irrelevant TCR and "soluble TCR free" controls both produced very low SLLMWITQC-HLA-A*0201 complex counts on the cell surface.

Claims:

1. An assay method comprising:

providing a plurality of test cells, which present a given TCR ligand,

contacting the test cells with an excess of αβ T cell receptors (TCRs) which specifically recognise and bind to said TCR ligand, said TCRs having a Kd for their interaction with the said TCR ligand of 50 nM or less,

said TCRs being labelled, or adapted to be labelled, with a detectable signal;

separating non-cell-bound TCRs from the cells,

in the case where the TCRs were adapted to be labelled rather than labelled, labelling the cell-bound TCRs with the detectable signal,

then detecting and quantifying the label signals from one or a plurality of the cells, and estimating from the resultant signal quantity(ies) the average number of said TCR ligand presented per cell.

2. A method as claimed in claim 1 wherein the TCR ligand is a Class I pMHC.

3. A method as claimed in claim 1 wherein the TCR ligand is a Class II pMHC.

4. A method as claimed in claim 1 wherein the TCR ligand is a CD1-antigen.

5. A method as claimed in any preceding claim wherein the label signals are detected and quantified cell by cell

6. A method as claimed in any preceding claim wherein the detectable signal is fluorescence.

7. A method as claimed in claim 6 wherein the label signals are detected by 3-dimensional fluorescence microscopy and quantified by counting individual fluorescent signals.

8. A method as claimed in any of the preceding claims wherein a suspension of cells is contacted with the TCR.

9. A method as claimed in any of claims 1 to 7 wherein cells are contacted with the TCR while adhered to a substrate.

10. A method as claimed in any of the preceding claims wherein the estimated average is adjusted by subtracting a number representing the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand.

11. A method as claimed in claim 10 wherein the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand is derived from one or more control assays wherein the method of any of claims 1 to 6 is repeated except that

(a) an analogous TCR which does not recognise the said TCR ligand is substituted for the cognate TCR, the resultant estimate being taken as representing the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand; and/or

(b) cells of the same cell classification but which do not present the said TCR ligand are substituted for the test cells, the resultant estimate being taken as representing the average number of TCRs bound to the cell otherwise than specifically to the TCR ligand.

Figure 1a

Clone 134A wt A6 TCR α chain DNA sequence

atgcagaaggaagtggagcagaactctggacccctcagtgttccagagggagccattg
cctctctcaactgcacttacagtgaccgaggttcccagtccttcttctggtacagaca
atattctgggaaaagccctgagttgataatgtccatatactccaatggtgacaaagaa
gatggaaggtttacagcacagctcaataaagccagccagtatgtttctctgctcatca
gagactcccagcccagtgattcagccacctacctctgtgccgttacaactgacagctg
ggggaaattgcagtttggagcagggacccaggttgtggtcaccccagatatccagaac
cctgaccctgccgtgtaccagctgagagactctaaatccagtgacaagtctgtctgcc
tattcaccgattttgattctcaaacaaatgtgtcacaaagtaaggattctgatgtgta
tatcacagacaaatgtgtgctagacatgaggtctatggacttcaagagcaacagtgct
gtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaacaacagcatta
ttccagaagacaccttcttccccagcccagaaagttcctaa

Figure 1b

Clone 134A wt A6 TCR α chain amino acid sequence

MQ
KEVEQNSGPL SVPEGAIASL NCTYSDRGSQ SFFWYRQYSG KSPELIMSIY
SNGDKEDGRF TAQLNKASQY VSLLIRDSQP SDSATYLCAV TTDSWGKLQF
GAGTQVVVTP DIQNPDPAVY QLRDSKSSDK SVCLFTDFDS QTNVSQSKDS
DVYITDKCVL DMRSMDFKSN SAVAWSNKSD FACANAFNNS IIPEDTFFPS
PESS*

Figure 2a

Clone 134 Mutated A6 TCR β chain DNA sequence

gctggtgtcactcagaccccaaaattccaggtcctgaagacaggacagagcatgacac
tgcagtgtgcccaggatatgaaccatgaatacatgtcctggtatcgacaagacccagg
catggggctgaggctgattcattactcagttggtgctggtatcactgaccaaggagaa
gtccccaatggctacaatgtctccagatcaaccacagaggatttcccgctcaggctgc
tgtcggctgctccctcccagacatctgtgtacttctgtgcctcgaggccggggctg**at**
gagtgcggaaccagagcagtacttcgggccgggcaccaggctcacggtcacagaggac
ctgaaaaacgtgttcccacccgaggtcgctgtgtttgagccatcagaagcagagatct
cccacacccaaaaggccacactggtgtgcctggccaccggtttctaccccgaccacgt
ggagctgagctggtgggtgaatgggaaggaggtgcacagtggggtctgcacagacccg
cagcccctcaaggagcagcccgccctcaatgactccagatacgctctgagcagccgcc
tgagggtctcggccaccttctggcaggacccccgcaaccacttccgctgtcaagtcca
gttctacgggctctcggagaatgacgagtggacccaggatagggccaaacccgtcacc
cagatcgtcagcgccgaggcctggggtagagcagactaa

Figure 2b

Clone 134A Mutated A6 TCR β chain amino acid sequence

MNAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQ
GEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASRPGL**MSAE**PEQYFGPGTRLTVT
EDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCT
DPQPLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKP
VTQIVSAEAWGRAD*

Figure 3

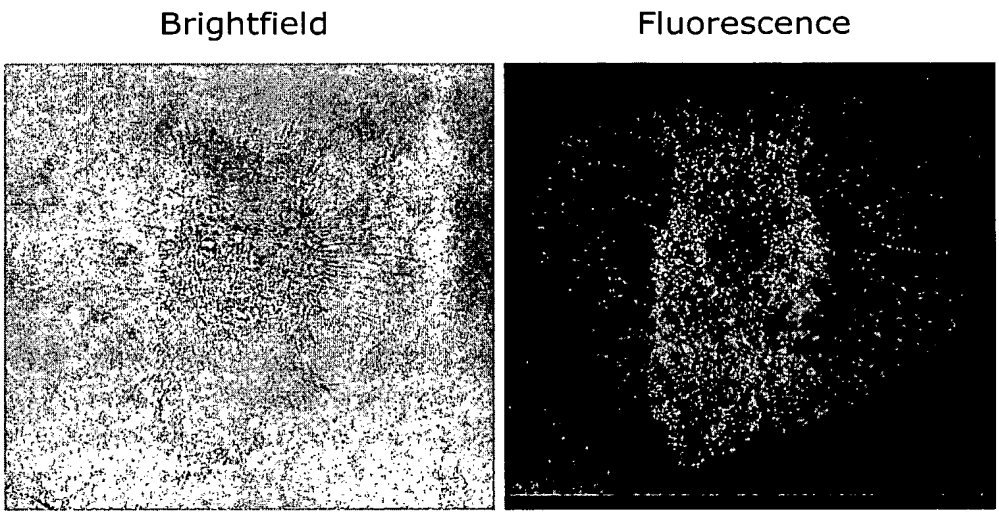
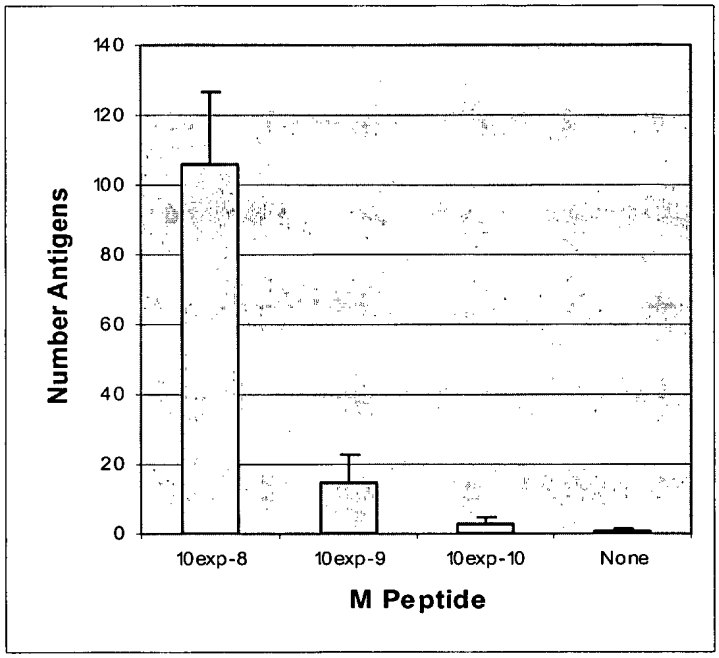


Figure 4



peptide [M]		10exp-8	10exp-9	10exp-10	None
Average		106	14.71429	2.465116	0.62069
N		6	35	43	28

Figure 5a

C58c61 alpha chain

MKQEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFR
QDPGKGLTSLLLITPWQREQTSGRLNASLDKSSGRSTLY
IAASQPGDSATYLCAVRPLLDGTYIPTFGRGTSLIVHPYI
QNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDS
DVYITDKCVLDMRSMDFKSNSAVAWSNKSDFACANAF
NNSIIPEDTFFPSPESS

Figure 5b

C58c61 beta chain

MNAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWY
RQDPGMGLRLIHYSVAIQTTDQGEVPNGYNVSRSTIEDF
PLRLLSAAPSQTSVYFCASSYLGNTGELFFGEGSRLTVL
EDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPD
HVELSWWVNGKEVHSGVCTDPQPLKEQPALNDSRYAL
SSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRA
KPVTQIVSAEAWGRAD

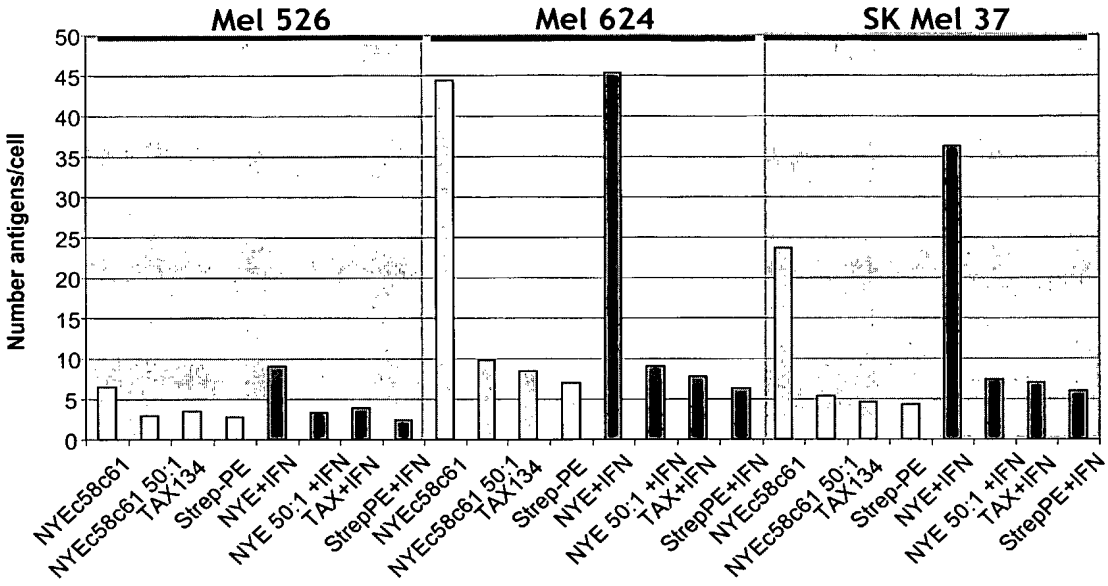
Figure 6a

atgaaacaggaggtgacgcagattcctgcagctctgagtgtcccagaaggagaaaact
tggttctcaactgcagtttcactgatagcgctatttacaacctccagtggtttaggca
ggaccctgggaaaggtctcacatctctgttgcttatt**acccc**c**gt**g**g**cagagagagcaa
acaagtggaagacttaatgcctcgctggataaatcatcaggacgtagtactttataca
ttgcagcttctcagcctggtgactcagccacctacctctgtgctgtgaggccc**ctttt**
gg**at**ggt**act**tacatacctacatttggaagaggaaccagccttattgttcatccgtat
atccagaacccggatcctgccgtgtaccagctgagagactctaagtcgagtgacaagt
ctgtctgcctattcaccgattttgattctcaaacaaatgtgtcacaaagtaaggattc
tgatgtgtatatcacagacaaatgtgtgctagacatgaggtctatggacttcaagagc
aacagtgctgtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaaca
acagcattattccagaagacaccttcttccccagcccagaaagttcctaa

Figure 6b

atgaatgctggtgtcactcagaccccaaaattccaggtcctgaagacaggacagagca
tgacactgcagtgtgcccaggatatgaaccatgaatacatgtcctggtatcgacaaga
cccaggcatggggctgaggctgattcattactcagttg**ct**a**tt**c**ag**a**c**cactgaccaa
ggagaagtccccaatggctacaatgtctccagatcaaccatagaggatttcccgctca
ggctgctgtcggctgctccctcccagacatctgtgtacttctgtgccagcagttacct
cgggaacaccggggagctgtttttggagaaggctctaggctgaccgtactggaggac
ctgaaaaacgtgttcccacccgaggtcgctgtgtttgagccatcagaagcagagatct
cccacacccaaaaggccacactggtgtgcctggccaccggtttctaccccgaccacgt
ggagctgagctggtgggtgaatgggaaggaggtgcacagtggggtctgcacagacccg
cagcccctcaaggagcagcccgccctcaatgactccagatacgctctgagcagccgcc
tgagggtctcggccaccttctggcaggacccccgcaaccacttccgctgtcaagtcca
gttctacgggctctcggagaatgacgagtggacccaggatagggccaaacccgtcacc
cagatcgtcagcgccgaggcctgggggagagcagactaa

Figure 7


Mel 526
Mel 624
SK Mel 37
Number antigens/cell
0
5
10
15
20
25
30
35
40
45
50
NYEc58c61
NYEc58c61 50:1
TAX134
Strep-PE
NYE+IFN
NYE 50:1 +IFN
TAX+IFN
StrepPE+IFN

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/003002

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/569 G01N33/58 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	GRATAMA J W ET AL: "Flow cytometric quantitation of immunofluorescence intensity: Problems and perspectives" CYTOMETRY 01 OCT 1998 UNITED STATES, vol. 33, no. 2, 1 October 1998 (1998-10-01), pages 166-178, XP002361539 ISSN: 0196-4763 pages 169-170 ----- -/--	1-6,10, 11

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 January 2006	24/01/2006

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax (+31-70) 340-3016	VON EGGELKRAUT, R

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/003002

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	HOLLER P D ET AL: "In vitro evolution of a T cell receptor with high affinity for peptide/MHC." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. 9 MAY 2000, vol. 97, no. 10, 9 May 2000 (2000-05-09), pages 5387-5392, XP002361464 ISSN: 0027-8424 cited in the application the whole document	1-6,10, 11
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Y	IRVINE DARRELL J ET AL: "Direct observation of ligand recognition by T cells." NATURE. 24 OCT 2002, vol. 419, no. 6909, 24 October 2002 (2002-10-24), pages 845-849, XP002361629 ISSN: 0028-0836 cited in the application page 848, column 2, paragraph MICROSCOPY	7-9
Y	----- WO 2004/044004 A (AVIDEX LIMITED; JAKOBSEN, BENT, KARSTEN; ANDERSEN, TORBEN, BENT; MOLLO) 27 May 2004 (2004-05-27) cited in the application pages 35-36; examples 24,25	4
A	example 25	1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2005/003002

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		CA 2505558 A1	27-05-2004
		EP 1558643 A2	03-08-2005